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13. ABSTRACT (Maximum 200 Words)
The purpose of this project was to address the critical question of identifying early genomic changes that occur in the mammary glands of patients with increased predisposition to breast cancer due to germ-line mutations in BRCA1/2 genes. Identification of these early changes will improve our understanding of the mechanisms of tumorigenensis in these high-risk patients and therefore should aid in devising early detection, diagnosis, and prevention strategies for such women at risk. Over the course of the project, we tested the hypothesis that genomic changes may be detected, not only in histologically abnormal and malignant breast tissues, but also in morphologically normal tissues as well as in areas with pathologically benign changes. We have clearly shown that genetic changes in areas with pathologically benign changes. We have clearly shown that genetic changes in the form of LOH are present in the non-malignant breast tissues in addition to the tumor, suggesting that these non-malignant tissues already harbor significant genetic alterations that may predispose them to malignant transformation. Our results support the hypothesis that there is a "field effect" of early genetic events preceding morphologic changes in the mammary glands of BRCA mutation carriers. To follow up on these findings we have recently initiated a study aiming at developing and testing molecular markers for early detection and diagnosis of hereditary breast cancer in BRCA1/2 carriers.

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Introduction:

Women who carry mutations in either *BRCA1* or *BRCA2* face markedly elevated risks of developing early onset breast cancer. Given the high risk of breast cancer in mutation carriers, the Cancer Genetics Studies Consortium has recommended that *BRCA1/2* carriers, and those with family histories consistent with hereditary breast cancer, have a mammogram performed on an annual basis, beginning between the ages of 25 and 35 (1). However, the optimal screening regimen in these high-risk women is not yet known. Studies have demonstrated that mammography has a relatively lower sensitivity in younger women (2). This lower sensitivity of mammography is presumed to be due to the greater breast radiodensity seen in younger women. Therefore, there is a critical need to develop screening methods to augment mammography for the early detection of breast tumors, particularly in high-risk women, such as *BRCA1* or *BRCA2* mutation carriers, who face markedly elevated risks of developing early onset breast cancer. One approach would be the use of molecular markers associated with neoplastic changes in a screening strategy for early detection. For this reason it is critical to identify such markers and therefore to study the molecular changes that occur early in the process of tumorigenesis.

BRCA1 and *BRCA2* are breast cancer susceptibility genes whose loss leads to defects in both the ability of a cell to repair DNA and to alter its gene transcriptional pattern (3, 4). It is likely that loss of both the DNA damage repair function and the role in transcriptional regulation contribute to the pathogenesis of breast cancer. In this study we will define the anatomical extent of *BRCA1* and *2* mutation induced changes in genetic integrity and gene transcription in order to identify markers that may be used for the early detection of *BRCA1/2* mutation related breast cancer. This study proposes to evaluate morphologically normal, proliferative, hyperplastic, and malignant tissues in surgical specimens from *BRCA1/2* mutation carriers for LOH at the *BRCA1* and *2* loci, respectively, and at the *FHIT* gene locus, as well as for evidence of other genetic abnormalities.

The onset of breast cancer involves transitions from normal mechanisms of cellular control of proliferation and survival to highly abnormal regulation of cellular processes as genomic instability increases. Inheritance of a defective allele of *BRCA1* or *BRCA2* predisposes mammary epithelial cells to loss of normal cellular control of growth and survival (5). In families that carry mutant alleles of one of these genes in their germ-lines, it is hypothesized that random errors in replication, recombination, mismatch, or excision repair lead to a loss or mutation of the wild-type allele. Although this type of pivotal genetic event is thought to mark the onset of the pathological changes that lead to breast cancer, it has not yet been demonstrated in human tissue from one of these predisposed individuals. This early event could be observed as LOH at an informative marker, within or in close linkage to the inherited gene in question. This initial LOH event may mark the onset of genomic instability and might facilitate the accumulation of further genetic damage and result in pathological changes within the tissue.

No studies to date have systematically examined the early consequences of inheritance of a mutation in the *BRCA1* or *BRCA2* genes for corresponding, early changes in breast histopathology. In addition, no studies have addressed the correlation of such early abnormalities in the breasts of *BRCA* mutation carriers with genomic gains, losses, loss of heterozygosity (LOH), or replication error repair instability. Early studies from

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several groups established that defects on chromosomes 13 and 17 were associated with familial breast cancer. Following the discovery of *BRCA1* on chromosome 17 and *BRCA2* on chromosome 13 (6, 7), more detailed mapping of defects on both chromosomes was carried out, primarily using LOH methodology. LOH of the two *BRCA* loci has been a highly reproducible observation in tumors of carriers of mutations in each respective gene(8, 9). However, several questions about the actual roles of these two genes in breast tumorigenesis remain unclear. For example, the basis of the vast variation in penetrance of different mutations in these two genes, variations of the same mutation in different individuals of the same family, and variations among families, are not clear. Our group has a long-standing interest in understanding the earliest steps in histopathologic changes and associated genomic alterations as breast cancer begin to arise in high risk, *BRCA*-mutation carrying individuals. The aim of our study is to provide a better understanding of early genomic alterations in high risk *BRCA1/2* carriers, and to determine whether these alterations correspond to specific histopathologic changes in the breast. Our results support the hypothesis that there is a "field effect" of genomic aberrations, in which some of the genetic changes detected in *BRCA*-associated cancers are also present in the non-malignant areas adjacent to the tumors, as well as in the *BRCA*-positive contralateral prophylactic mastectomy specimens.

Body:

We have successfully completed all the tasks that we proposed in this project, and we were able to test our hypothesis and show and characterize a "field effect" of genetic changes in the mammary glands of *BRCA1/2* patients involving non-tumor tissues.

- We have recruited Dr. Luciane Cavalli, a postdoctoral fellow with an excellent experience in cytogenetics and molecular biology, to work on this project, in Dr. Haddad's lab.

- Study participants were accrued through the Familial Cancer Registry (FCR), a shared resource of the Lombardi Cancer Center (LCC). The FCR includes over 1300 individuals with familial or hereditary breast cancer. Members of the FCR complete questionnaires, which include detailed information on their medical history, family history, breast and ovarian cancer risk factors, and other comprehensive epidemiological data. These data are updated on an annual basis. In addition, if these women have undergone any surgery, be it prophylactic or for diagnostic or treatment purposes, the tissue is obtained and stored. Blood is also obtained from these patients and the lymphocytes are immortalized. DNA is prepared and banked. The collection of familial breast tumors and mastectomy specimens is archived in the LCC Histopathology and Tissue Shared Resource.

- Pathology review of the *BRCA* 1/2 positive patients: Working with Dr. Baljit Singh, an expert breast pathologist, we have examined benign breast tissue from archival material from 21 cases with *BRCA1/2* mutations and from 24 cases of familial breast carcinoma without *BRCA* mutations, from our familial cancer registry (FCR).[95] We have shown that *BRCA* mutants had sclerosing adenosis in 76% of cases (16/21),

usual ductal hyperplasia (UDH) in 38%, small duct papilloma in 10% and radial scar in 5%. Cases of familial breast carcinoma without *BRCA* mutations had sclerosing adenosis in 50% (12/24), UDH in 8%, atypical ductal hyperplasia in 4%, radial scar in 12.5% cases. 12% (2/17) of cases with *BRCA* mutants predominantly had lobules type I and 88% of the cases contained lobules, type II. Terminal ductular lobular units (lobules) have been categorized as lobules I, II, III, based on the expression patterns of estrogen and progesterone receptors, and the rate of proliferation, as measured by Ki-67 immunohistochemistry (10). The highest percentages of these markers are found in lobules type I. Lobules type I are seen at higher rates in association with ductal hyperplasia and sclerosing adenosis, and it has been postulated that ductal hyperplasia may arise from these structures. Figures 1A and 1C show examples of a morphologically normal lobule (1A) and sclerosing adenosis (1C), both from areas adjacent to a breast carcinoma from a *BRCA1* positive patient in this group. Thus we have demonstrated that breast cancer patients with *BRCA1* and *BRCA2* mutations have a higher incidence of proliferative breast disease than the non-carriers. A higher incidence of usual ductal hyperplasia was seen in *BRCA* mutants than non-mutants. However a higher incidence of lobules type I was not seen in the *BRCA* mutants. These data were presented at the 2001 United States and Canadian Academy of Pathology Annual Meeting. (See Reportable Outcomes section and appendix).

- Laser capture microdissection (LCM): Dr. Cavalli was trained to use the LCM system which is available at the LCC Histopathology and Tissue Shared Resource. She has spent several hours of training using sporadic breast tumor samples available from the LCC tumor bank and also worked with members of the NIH LCM core facility, directed by Drs. Robert Bonner and Lance Liota, where this technique was developed. She has visited the facility at NIH and had the chance to interact with the core staff and discuss the protocol they follow. Members of our research team have also been attending each year the LCM symposium held at NIH where several presentations and discussions about this approach take place. This yearly meeting allows very helpful interactions to take place among users and experts in the field.

- We have established a panel of polymorphic dinucleotide microsatellite markers, including markers for the *BRCA1* gene (intragenic markers and closely linked markers), for other loci on chromosome 17, for the *BRCA2* gene (closely linked markers), for other loci on chromosome 13, and for the *FHIT* gene on 3p14.2 (intragenic markers) (table 1). These markers were chosen based on their reported high heterozygosity rate (11-13). We have established and validated the experimental conditions to evaluate each of the markers in a reliable and reproducible way, using DNA prepared from laser capture microdissected (LCM) specimens. This step is critical for our study, as it allows us to select specific epithelial cells for molecular analysis following accurate histologic characterization by the pathologist.

- For each patient studied, the markers are 1st evaluated using DNA prepared from the patient's blood to identify the markers which are informative. Breast tissue sections are carefully evaluated by our expert breast pathologist, Dr. Singh, who marks each section to clearly indicate the malignant and premalignant tissues, and the

morphologically normal looking tissues surrounding the tumor, and the areas with benign changes such as sclerosing adenosis (SA), usual ductal atypia, or atypical ductal hyperplasia. LCM is then used to isolate cells from the different areas described above. LOH analysis is then performed on DNA isolated from each area.

- Using the informative polymorphic dinucleotide microsatellite markers, we performed a total of 105 analyses at different loci in areas showing either normal TDLUs or benign proliferative changes such as sclerosing adenosis; of these, LOH was detected in 59 studies (56%). In the normal tissues, 15 of 30 analyses (50%) showed LOH, and in the tissues with proliferative changes, 44 of 75 analyses (59%) showed LOH. Table 2 summarizes the results and Figures 1 and 2 show examples of LOH studies. These data were presented at the 2002 AACR annual meeting and the 2002 Era of Hope meeting. They were also summarized in a paper in press (20). (See Reportable Outcomes section and appendix).

Table 1. The panel of microsatellite markers used. The chromosomal location, the expected size range of the amplified PCR product and the percent heterozygozyty for ecach marker are indicated.

	Site	Size (bp)	Heterozygozity (%)
D17S786	17p12	135-157	77
TP 53	17p13	230	90
D17S849	17p13	215-253	67
D17S250	17q11.2-q12	151-169	91
D17S806	17q21	153-185	91
D17S855	17q21.2 (*BRCA1*)	145	82
D17S579	17q21.3	111-133	87
D17S785	17q24	181-207	84
D17S784	17q25	226-238	79
D13S289	13q12.1	260-276	74
D13S153	13q14.1-q14.3	212-236	82
D13S137	13q14.3	113-135	84
D13S173	13q32-q34	166-178	84
D3S1300	3p21.1-14.2 (*FHIT*)	217-241	83
D3S1481	3p14.2 (*FHIT*)	104	83

Table 2. Summary of the results showing the number of analyses performed and the percent of LOH detected.

	Total # of analyses	LOH found
Total	105	59 (56%)
Normal TDLU	30	15 (50%)
Proliferative Changes	75	44 (59%)

Figure 1: The left top image shows a histologically normal terminal duct lobular unit (H&E, 20X) adjacent to the tumor, from a *BRCA1* positive patient with cancer. The left middle image shows the lobule after laser capture microdissection (LCM). A majority of the epithelial tissue has been microdissected. The left bottom image shows an area with sclerosing adenosis (arrow head) (H&E, 20X), adjacent to the tumor from the same patient. The tracings on the right show the LOH analysis using the D17S855 *BRCA1* intragenic marker, performed on tissues microdissected from the same patient's specimens. Tissues studied were isolated using LCM. From top to bottom, studies in blood (Bl) show the marker to be heterozygote. Loss of heterozygosity is detected in the tumor (T). The same allele is also lost in normal lobular tissue adjacent to the tumor (N1) and in an area with sclerosing adenosis adjacent (SA1) to the tumor. In the contralateral breast, normal tissues (N2) do not show LOH, while an area with sclerosing adenosis (SA2) shows LOH for the *BRCA1* marker.
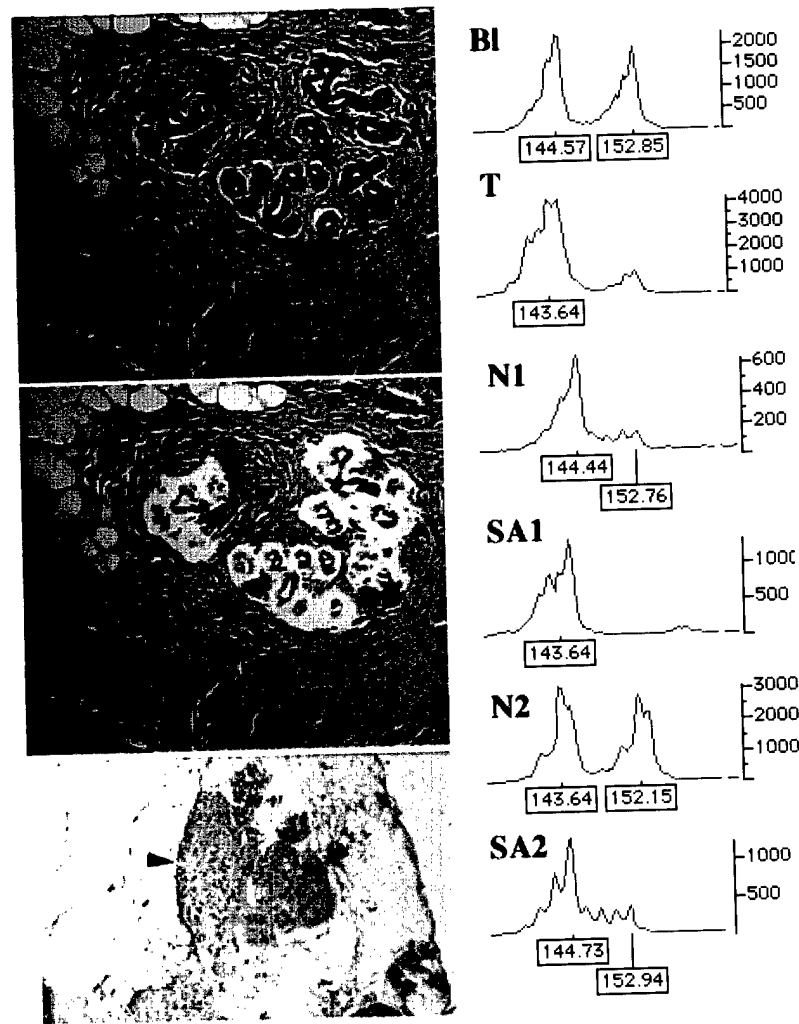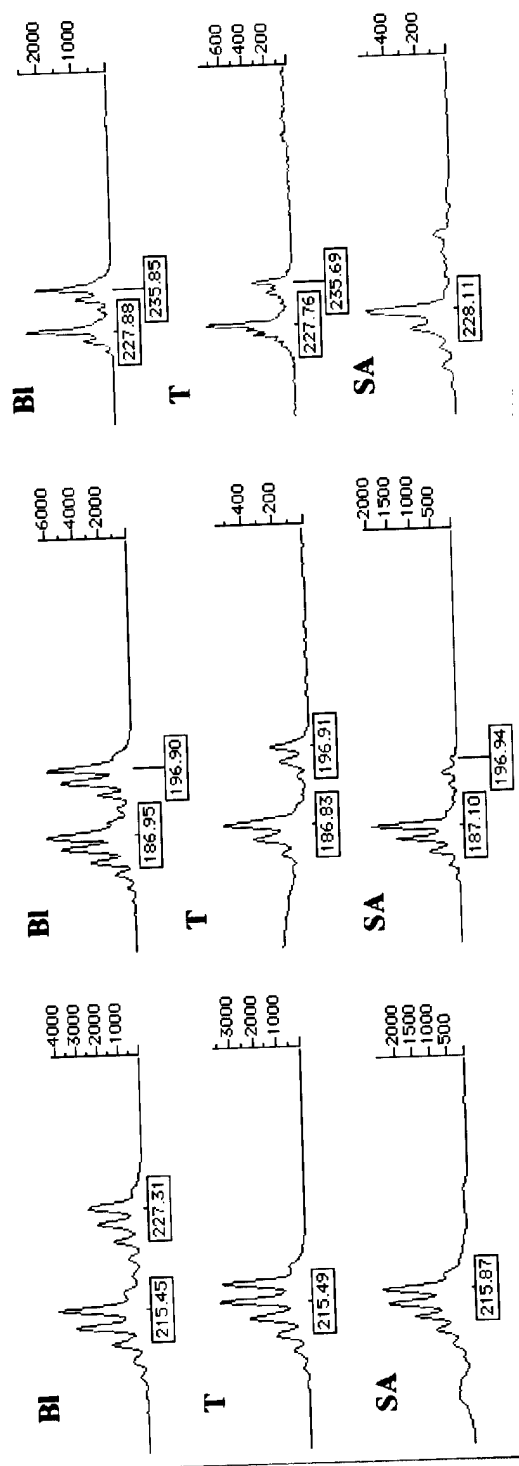
Figure 2: Figure 2 shows other examples of LOH at the following loci (from left to right): D13S153, D17S785, and D3S1300 in cases 5, 4, and 3, respectively. In each panel, the top tracing shows the analysis in the blood (Bl), the middle tracing shows the analysis in the tumor (T) and in the bottom tracing the analysis in an area with sclerosing adenosis.

Key Research Accomplishments:

- Morphologic characterization of breast tissue specimens from *BRCA1/2* carriers and comparison of the findings in this group to the findings in breast tissues from non-carriers.
- Development of a panel of microsatellite markers to study LOH on chromosomes 13, 17 and 3p in laser capture microdissected (LCM) specimens.
- LOH analysis of morphologically normal breast tissues and breast tissues with benign changes, carefully microdissected from *BRCA1/2* positive patients with breast cancer.
- Description of a field effect of genetic aberrations which extends beyond the tumor to include areas with normal and benign morphology.

Revised and approved statement of work:

Year 1: In the first year, we will obtain hereditary breast tumors with associated mastectomy tissue as well as prophylactic mastectomies from *BRCA* carriers. [Completed]. We will also fully establish and validate all necessary LOH assays, following pathologic review of all specimens, for comparison of their genomic changes relative to nearby pathologically reviewed and microdissected non-tumor tissue (Aims 1 and 2). [Completed].

Year 2: In the second year, specimen collection will continue, Aims 1 and 2 will continue, and Aims 3 and 4 (study of pathologically reviewed contralateral prophylactic mastectomy tissues and pathologically reviewed bilateral prophylactic mastectomy tissues) will begin. [Completed].

Year 3: In the third year, all 4 aims will be completed and data analyzed. Specifically, pathologic diagnosis will be correlated with genomic and chromosomal changes for each aim. [Completed].

Reportable Outcomes:

- Manuscript accepted for publication: Loss of Heterozygosity in Normal Breast Epithelial Tissue and Benign Breast Lesions in *BRCA1/2* Carriers with Breast Cancer. Cavalli LR, Singh B, Isaacs C, Dickson RB, and Haddad BR. <u>Cancer Genet Cytogenet</u> *in press*.

- Abstract and Poster presentation at the United States and Canadian Academy of Pathology Annual Meeting, 2001: Spectrum of Benign Breast Disease in Familial Breast Cancer Patients With and Without BRCA Mutations. B. Singh, L.Seltzer, M. Ossandon, S. Constable, M. Joyner, B. Haddad, J. Stead, C. Lerman, C. Isaacs.

- Abstract and Platform presentation at the American Association for Cancer Research Annual Meeting, 2002: Evidence for Genomic Instability in Morphologically Normal Breast Tissues and in Benign Breast Lesions in *BRCA1/2* Positive Patients with Breast Cancer. Cavalli, LR., Singh, B., Isaacs C., Dickson RB., and Haddad, BR.

- Abstract and Platform presentation at the Era of Hope 2002 meeting: Evidence for Genomic Instability in Morphologically Normal Breast Tissues and in Benign Breast Lesions in *Brca1/2* Positive Patients with Breast Cancer. Haddad, BR., Cavalli, LR., Singh, B., Isaacs C., and Dickson RB.

- Data was used to support in part a patent application under consideration by the US patent office entitled: Methods for Early Detection of Cancer. Inventors: Haddad, BR., Dickson, RB., Isaacs C, and Cavalli, LR.

Conclusion:

This is the first study to demonstrate genetic changes (LOH) in normal terminal ductal lobular units (TDLUs) and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients. In this study, we have detected LOH at multiple loci, not only in malignant regions in the breasts of *BRCA1* and *BRCA2* mutation carriers with breast cancer, but also in morphologically normal TDLUs and in tissues with benign proliferative changes (e.g. sclerosing adenosis) from these patients. Most of these losses, detected in the normal and benign tissues, were also present in the tumor tissues, suggesting that these non-malignant tissues already harbor significant genetic alterations that may predispose to malignant transformation. Our results support the hypothesis that there is a "field effect" of genomic aberrations, in which some of the genetic changes detected in *BRCA*-associated cancers are also present in the non-malignant areas adjacent to the tumors, as well as in the *BRCA*-positive contralateral prophylactic mastectomy specimens. The concept of "field cancerization" was originally described by Slaughter *et al* in 1953 (14) in their study of oral cancer and was used in reference to the presence of pre-neoplastic histologic changes at multiple sites. More recent studies have described the molecular changes associated with the multistep development and progression of cancer (15). In several other studies, morphologically "normal tissues" adjacent to tumors have been shown to harbor molecular changes such as LOH, microsatellite alterations (16, 17), chromosomal instability (18), and mutations in the *TP53* gene (19). Such studies elegantly provide a molecular definition of the old concept of "field cancerization", and may indicate the presence of a field of genetically altered cells which may be at higher risk of cancer transformation.

We conclude that LOH at the relevant *BRCA* loci is an early event in *BRCA* mutation carriers, and may be detected in non-malignant cells. The significance of this finding needs further evaluation in larger studies, and ultimately may be used as a marker for elevated risk of malignant transformation in these high-risk patients. The identification of such early genetic changes will improve our understanding of the mechanisms of tumorigenesis, and may be useful to develop molecular markers for early detection and diagnosis of hereditary breast cancer in *BRCA1/2* carriers. In order to follow up on these findings, we have recently initiated a study aiming at developing and testing molecular markers for early detection and diagnosis of hereditary breast cancer in *BRCA1/2* carriers.

References:

1. Burke, W., M. Daly, J. Garber, J. Botkin, M.J. Kahn, P. Lynch, et al. 1997. *Recommendations for follow-up care of individuals with an inherited predisposition to cancer. II. BRCA1 and BRCA2. Cancer Genetics Studies Consortium [see comments].* Jama, 277(12): p. 997-1003.
2. Tabar, L., G. Fagerberg, S.W. Duffy, N.E. Day, A. Gad, and O. Grontoft. 1992. *Update of the Swedish two-county program of mammographic screening for breast cancer.* Radiol Clin North Am, 30(1): p. 187-210.
3. Chen, Y., W.H. Lee, and H.K. Chew. 1999. *Emerging roles of BRCA1 in transcriptional regulation and DNA repair.* J Cell Physiol, 181(3): p. 385-92.
4. Deng, C.X. and S.G. Brodie. 2000. *Roles of BRCA1 and its interacting proteins.* Bioessays, 22(8): p. 728-37.
5. Ingvarsson, S. 1999. *Molecular genetics of breast cancer progression.* Semin Cancer Biol, 9(4): p. 277-88.
6. Miki, Y., J. Swensen, D. Shattuck-Eidens, P.A. Futreal, K. Harshman, S. Tavtigian, et al. 1994. *A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1.* Science, 266(5182): p. 66-71.
7. Wooster, R., G. Bignell, J. Lancaster, S. Swift, S. Seal, J. Mangion, et al. 1995. *Identification of the breast cancer susceptibility gene BRCA2.* Nature, 378(6559): p. 789-92.
8. Cornelis, R.S., S.L. Neuhausen, O. Johansson, A. Arason, D. Kelsell, B.A. Ponder, et al. 1995. *High allele loss rates at 17q12-q21 in breast and ovarian tumors from BRCA1-linked families. The Breast Cancer Linkage Consortium.* Genes Chromosomes Cancer, 13(3): p. 203-10.
9. Gudmundsson, J., G. Johannesdottir, A. Arason, J.T. Bergthorsson, S. Ingvarsson, V. Egilsson, et al. 1996. *Frequent occurrence of BRCA2 linkage in Icelandic breast cancer families and segregation of a common BRCA2 haplotype.* Am J Hum Genet, 58(4): p. 749-56.
10. Russo, J., D. Reina, J. Frederick, and I.H. Russo. 1988. *Expression of phenotypical changes by human breast epithelial cells treated with carcinogens in vitro.* Cancer Res, 48(10): p. 2837-57.
11. Kerangueven F., Noguchi T., Coulier F., Allione F., Wargniez V., Simony-Lafontaine J., Longy M., Jacquemier J., Sobol H., Eisinger F., and Birnbaum D. Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. Cancer Res. 57:5469-5474, 1997.
12. Phelan C.M., Borg A., Cuny M., Crichton D.N., Baldersson T., Andersen T.I., Caligo M.A., Lidereau R., Lindblom A., Seitz S., Kelsell D., Hamann U., Rio P., Thorlacius S., Papp J., Olah E., Ponder B., Bignon Y.J., Scherneck S., Barkardottir R., Borresen-Dale A.L., Eyfjord J. Theillet C., Thompson A.M., Devilee P., and Larsson C. Consortium study on 1280 breast carcinomas: allelic loss on chromosome 17 targets subregions associated with family history and clinical parameters. Cancer Res.58:1004-1012, 1998.
13. Euhus D.M., Maitra A., Wistuba II., Alberts A., Albores-Saavedra J., and Gazdar A.F. Loss of heterozygosity at 3p in benign lesions preceding invasive breast cancer. J.Surg.Res.83:13-18, 1999.

14. Slaughter D.P., Southwick, H.W., and Smejkal, W. "Field cancerization" in oral stratified squamous epithelium. Cancer (Phila.), *6*:693-968, 1953.
15. Fearon E.R. and Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. *61*:759-767, 1990.
16. Deng G., Lu Y., Zlotnikov G., Thor A.D., and Smith H.S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science *274*:2057-2059, 1996.
17. Tabor M.P., Brakenhoff R.H., van Houten V.M., Kummer J.A., Snel M.H., Snijders P.J., Snow G.B., Leemans C.R., and Braakhuis B.J. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. Clin Cancer Res. *7*:1523-1532, 2001.
18. Hittelman W.N. Genetic instability in epithelial tissues at risk for cancer. Ann N Y Acad Sci. *952*:1-12, 2001.
19. Brennan J.A., Mao L., Hruban R.H., Boyle J.O., Eby Y.J., Koch W.M., Goodman S.N., and Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med. *16*;332(7):429-435, 1995.
20. Cavalli LR, Singh B, Isaacs C, Dickson RB, and Haddad BR. Normal Breast Epithelial Tissue and Benign Breast Lesions in *BRCA1/2* Carriers with Breast Cancer. Cancer Genet Cytogenet *in press*.

Personnel:

This is a project which was completed due to a collaborative effort of a team of Georgetown University/Lombardi Cancer Center Researchers including, Drs. Bassem Haddad (PI) and Robert Dickson, Baljit Singh and Luciane Cavalli (Post-Doctoral Fellow).

Bibliography of all Publications:

- Cavalli LR, Singh B, Isaacs C, Dickson RB, and Haddad BR. Normal Breast Epithelial Tissue and Benign Breast Lesions in *BRCA1/2* Carriers with Breast Cancer. <u>Cancer Genet Cytogenet</u> *in press.*

- B. Singh, L.Seltzer, M. Ossandon, S. Constable, M. Joyner, B. Haddad, J. Stead, C. Lerman, C. Isaacs. Spectrum of Benign Breast Disease in Familial Breast Cancer Patients With and Without BRCA Mutations. Abstract. The United States and Canadian Academy of Pathology Annual Meeting, 2001.

- Cavalli, LR., Singh, B., Isaacs C., Dickson RB., and Haddad, BR. Evidence for Genomic Instability in Morphologically Normal Breast Tissues and in Benign Breast Lesions in *BRCA1/2* Positive Patients with Breast Cancer. Abstract. The American Association for Cancer Research Annual Meeting, 2002.

- Haddad, BR., Cavalli, LR., Singh, B., Isaacs C., and Dickson RB. Evidence for Genomic Instability in Morphologically Normal Breast Tissues and in Benign Breast Lesions in *Brca1/2* Positive Patients with Breast Cancer. Abstract. The Era of Hope 2002 meeting.

Appendices:

- Abstract to the United States and Canadian Academy of Pathology Annual Meeting, 2001.

- Abstract to the American Association for Cancer Research Annual Meeting, 2002.

- Abstract to the Era of Hope 2002 meeting.

- Manuscript in press in <u>Cancer Genet Cytogenet</u>

Spectrum of Benign Breast Disease in Familial Breast Cancer Patients With and Without BRCA Mutations. B. Singh, L.Seltzer, M. Ossandon, S. Constable, M. Joyner, B. Haddad, J. Stead, C. Lerman, C. Isaacs Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC

Background: The pathology of familial breast carcinoma with and without BRCA mutations has been reported. However, a systematic review of benign breast disease has hitherto not been performed in this cohort of patients.

Design: Hematoxylin and Eosin stained slides from formalin fixed, paraffin embedded archival tissue from 20 cases with BRCA1/2 mutations and 12 cases of familial breast carcinoma without BRCA mutations were examined. One representative slide from of benign breast disease from seventeen patients with BRCA mutations was immunostained for estrogen, progesterone receptors and Ki67 to categorize the proliferative status of the terminal duct lobular units and classify them as Lobule type I, II or III.

Results: BRCA mutants had sclerosing adenosis in 76% (16/21), usual ductal hyperplasia (UDH) in 38%, small duct papilloma in 10% and radial scar in 5% cases. Cases of familial breast carcinoma without BRCA mutations had sclerosing adenosis in 50% (12/24), UDH in 8%, atypical ductal hperplasia in 4%, radial scar in 12.5% cases. 12% (2/17) of cases with BRCA mutants predominantly had lobules type I and 88% cases contained lobules, type II.

Conclusion: Proliferative fibrocystic changes were observed in 76% of cases with BRCA mutations and in 50% of cases with familial breast carcinoma without BRCA mutations. The frequency of proliferative changes in BRCA mutants is similar to that reported in sporadic breast carcinoma patients and is less frequent in non-mutants. The lobules in BRCA mutants, all of which were parous women, revealed low proliferative activity as assessed by Ki67 staining.

Abstract presented at the 2002 AACR annual meeting:

Evidence for Genomic Instability in Morphologically Normal Breast Tissues and in Benign Breast Lesions in *BRCA1/2* Positive Patients with Breast Cancer. Cavalli, LR., Singh, B., Isaacs C., Dickson RB., and Haddad, BR.

Because many of hereditary breast cancer patients carry a mutation in *BRCA1* on chromosome 17 or *BRCA2* on chromosome 13, the first genetic event that may occur in their mammary glands to begin the carcinogenic progression toward cancer may be loss of heterozygosity (LOH) on one of these two chromosomes. It is unknown if these genetic changes correspond to a recognizable histopathological abnormality, or at what stage of the disease progression they occur. In this study, we evaluated the early genomic changes that occur in the mammary glands of patients with increased predisposition to breast cancer due to germ-line mutations in the *BRCA1/2* genes. We tested the hypothesis that these genomic changes may be detected not only in the histologically abnormal and malignant regions of these high risk women, but also in morphologically normal looking tissues and in areas with benign changes. Using a panel of 15 microsatellite markers we have evaluated 23 areas showing normal morphology or benign changes, such as sclerosing adenosis, from four *BRCA1* and one *BRCA2* positive patients with breast cancer. For each case, tissue sections were carefully evaluated by the pathologist and areas with morphologically normal tissues surrounding the tumor and areas with benign changes such as sclerosing adenosis were marked and microdissected using Laser Capture Microdissection (LCM) technology prior to LOH analysis. LOH was detected in both morphologically normal tissues and tissues with benign changes from all the cases investigated. Allelic losses of a minimum of 2 different markers, were observed in 17 of 23 areas with normal morphology or benign changes (Sclerosing Adenosis). In a *BRCA1* case, a sequential loss of two markers on chromosome 17q, previously observed in the tumor, was observed in morphologically normal looking areas up to 8mm distant from the tumor, as well, as in the other breast quadrants from the same breast where the tumor was present. An interesting finding was the detection of LOH in the normal contralateral breast removed prophylactically from this patient. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients. These data will aid in improved early detection and diagnosis of hereditary breast cancer and provide more information when considering prevention strategies for such women at risk.

Abstract presented at the 2002 Era of hope meeting:

EVIDENCE FOR GENOMIC INSTABILITY IN MORPHOLOGICALLY NORMAL BREAST TISSUES AND IN BENIGN BREAST LESIONS IN BRCA1/2 POSITIVE PATIENTS WITH BREAST CANCER

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Because many of hereditary breast cancer patients carry a mutation in BRCA1 on chromosome 17 or BRCA2 on chromosome 13, the first genetic event that may occur in their mammary glands to begin the carcinogenic progression toward cancer may be loss of heterozygosity (LOH) on one of these two chromosomes. It is unknown if these genetic changes correspond to a recognizable histopathological abnormality, or at what stage of the disease progression they occur. In this study, we evaluated the early genomic changes that occur in the mammary glands of patients with increased predisposition to breast cancer due to germ-line mutations in the BRCA1/2 genes. We tested the hypothesis that these genomic changes may be detected not only in the histologically abnormal and malignant regions of these high risk women, but also in morphologically normal looking tissues and in areas with benign changes. Using a panel of 15 microsatellite markers we have evaluated 23 areas showing normal morphology or benign changes, such as sclerosing adenosis, from four BRCA1 and one BRCA2 positive patients with breast cancer. For each case, tissue sections were carefully evaluated by the pathologist and areas with morphologically normal tissues surrounding the tumor and areas with benign changes such as sclerosing adenosis were marked and microdissected using Laser Capture Microdissection (LCM) technology prior to LOH analysis. LOH was detected in both morphologically normal tissues and tissues with benign changes from all the cases investigated. Allelic losses of a minimum of 2 different markers, were observed in 17 of 23 areas with normal morphology or benign changes (Sclerosing Adenosis). In a BRCA1 case, a sequential loss of two markers on chromosome 17q, previously observed in the tumor, was observed in morphologically normal looking areas up to 8mm distant from the tumor, as well, as in the other breast quadrants from the same breast where the tumor was present. An interesting finding was the detection of LOH in the normal contralateral breast removed prophylactically from this patient. Such changes may represent the earliest detectable genomic aberrations that occur during the development and progression of breast cancer in these high-risk patients and may aid in improved early detection.

Loss of Heterozygosity in Normal Breast Epithelial Tissue and Benign Breast Lesions in *BRCA1/2* Carriers with Breast Cancer

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Abbreviated title: LOH in normal and benign breast tissues from *BRCA1/2* carriers

ABSTRACT

Loss of heterozygosity (LOH) of the wild type *BRCA1/2* allele is a reproducible event in breast tumors of *BRCA1/2* mutation carriers. However, it is currently unknown if this allelic loss occurs only in association with recognizable histopathological abnormalities. In this study, we evaluated the early genomic changes that occur in the mammary glands of patients with increased predisposition to breast cancer due to germ-line mutations in the *BRCA1/2* genes. We tested the hypothesis that these genomic changes may be detected, not only in histologically abnormal and malignant breast tissues, but also in morphologically normal tissues as well as in areas with pathologically benign changes. Samples were obtained from five breast cancer patients: four *BRCA1* carriers and one *BRCA2* carrier. In each case, non-tumor tissue areas surrounding the tumor or from other locations of the breast were isolated using laser capture microdissection (LCM). We evaluated 29 areas showing normal terminal ductal lobular units (TDLU) or histopathologically benign changes (in particular sclerosing adenosis), using a panel of polymorphic dinucleotide microsatellite markers for the *BRCA1* gene and other chromosome 17 loci, for the *BRCA2* gene and other chromosome 13 loci, and for the *FHIT* gene on chromosome 3p14.2. Overall, we performed a total of 105 analyses; LOH was detected in 59 of the 105 studies (56%). In the normal TDLUs, 15 of 30 analyses (50%) showed LOH, and in the tissues with proliferative changes, such as sclerosing adenosis, 44 of 75 analyses showed LOH (59%). Our results suggest that there is a "field effect" of early genetic events preceding morphologic changes in the mammary glands of *BRCA* mutation carriers.

INTRODUCTION

The onset and progression of breast cancer involves progressive deviations from normal control mechanisms of cellular proliferation, followed by selection and highly abnormal regulation of both processes as genomic instability increases. Breast carcinomas are known to display a high degree of intratumor genetic heterogeneity, without any obvious correlation to their morphological appearance. Breast cancer progression is associated with genomic instability where loss of heterozygosity (LOH) is a key mechanism of inactivation of the wild type allele, corresponding to recessive, loss-of function mutations in tumor suppressor genes [1]. Because many of the patients with hereditary breast cancer carry a mutation in one of two known tumor suppressor genes, *BRCA1* on chromosome 17 or *BRCA2* on chromosome 13, LOH of the wild type *BRCA* allele may be the first genetic event that occurs in the mammary glands of these patients beginning carcinogenic progression [2-5]. Although this type of pivotal initial genetic event is likely due to the highly reproducible observation of *BRCA* LOH in tumors of carriers of *BRCA* mutations, any correlation of the initial LOH event with specific, early histopathologic change(s) remains completely unknown. If *BRCA* genes are haploid sufficient and act as gatekeepers of genetic alterations in mammary epithelial cells, then LOH of *BRCA* loci would be expected to correlate with onset of an early epithelial breast abnormality(s) and would be evidence of genetic instability in *BRCA* carrier. So far, however, *BRCA1* has been demonstrated to be haploid sufficient only in the context of embryonic fibroblasts of mice bearing a knockout of one of its alleles [6]. An early manifestation of genomic instability in human breast cancer may promote further LOH and gene amplification, such as amplification of *c-ERBB2* and *c-MYC*, since these

alterations seem to occur throughout all stages of the disease, from ductal carcinoma *in situ* (DCIS) onward.

Other studies have shown an alteration or reduced expression of the Fragile Histidine Triad (*FHIT*) gene on 3p14.2 in a significant fraction of breast carcinomas [7], and an association between these changes and breast tumor progression, suggesting that *FHIT* may have suppressor-like properties [8, 9]. In familial breast cancer kindreds, early studies [10] showed a high frequency of allelic imbalance at 3p14 relative to the frequency of allelic imbalance in the same region in sporadic breast cancers. More recent studies in *BRCA2* positive tumors have demonstrated frequent *FHIT* LOH and reduced expression of the FHIT protein. [11, 12].

The aim of our study is to provide a better understanding of early genomic alterations in high risk *BRCA1/2* carriers, and to determine whether these alterations correspond to specific histopathologic changes in the breast. One approach is to determine whether a "field effect" of genetic changes is present in the absence of histopathologic changes, i.e. in normal and benign tissues in the same breast with a *BRCA* associated cancer. A second approach is to study the same changes in prophylactic mastectomy specimens from *BRCA1* carriers. We used Laser Capture Microdissection (LCM) to selectively isolate pure mammary epithelial cell populations from 29 TDLUs and from areas with benign proliferative changes (e.g. sclerosing adenosis) from *BRCA1* carriers. In these studies, we examined both specimens with tumors and specimens from prophylactic mastectomies. The samples were evaluated for LOH using polymorphic dinucleotide microsatellite markers. These included intragenic and closely linked markers for the *BRCA1*gene, for other loci on chromosome 17, for the *BRCA2* gene (closely

linked markers), for other loci on chromosome 13, and for the *FHIT* gene (intragenic) on chromosome 3p14.2. Overall, we performed a total of 105 analyses at different loci; of these, LOH was detected in 59 studies (56%). This is the first study that demonstrates LOH at the *BRCA1/2* loci and the *FHIT* gene in non-malignant mammary epithelial tissue of *BRCA1/2* mutation carriers. Our results suggest that *BRCA* LOH is an early genetic change in the mammary epithelial cells of these high-risk patients, and that this change is associated with sclerosing adenosis.

MATERIAL AND METHODS

Specimen availability:

Specimens were obtained from five patients of defined *BRCA* status enrolled in the Familial Cancer Registry (Lombardi Cancer Center, Georgetown University): four *BRCA1* and one *BRCA2* mutation carriers. DNA was extracted from peripheral blood and breast tissue samples. All patients had invasive ductal carcinoma. In one case (case 1), a contralateral prophylactic masetectomy specimen was also available for analysis in additon to the breast with tumor. In another case (case 3), the patient had a lumpectomy for invasive ductal carcinoma, followed by mastectomy due to positive surgical margins. The mastectomy specimen showed foci of DCIS with no evidence of ductal carcinoma. Both specimens were available for analysis.

Histopathological Analysis:

The formalin fixed paraffin embedded tissues from the above specimens were examined by an expert breast pathologist (BS). Normal terminal ductal lobular units (TDLU) were defined as compact lobules with less than 20 lobular buds. Lobules were categorized as sclerosing adenosis when they were double or more than double the size of the normal TDLUs, and typically had increased lobules and a central area of fibrosis.

Laser capture microdissection (LCM):

LCM was utilized to obtain pure cell populations of selected areas from formalin-fixed-paraffin embedded tissue sections. Representative slides were reviewed by the pathologist (BS) and areas of normal TDLUs and sclerosing adenosis were delineated. Consecutive sections were carefully microdissected using a Pix Cell laser capture microscope (Pix Cell – Arcturus Engineering, Mountain View, CA) as described

previously [13]. On the average, 2000 cells were isolated from each designated area (Figure 1).

A total of 29 areas showing normal TDLUs (10 areas), or benign proliferative changes such as sclerosing adenosis (19 areas), in addition to the tumor were evaluated for evidence of LOH. The non-tumor tissues analyzed were microdissected using LCM, from areas adjacent to the tumor (same quadrant), from other quadrants of the same breast, and in one case (*BRCA1* mutation carrier), from the prophylactic mastectomy specimens of the contralateral normal breast.

Loss of heterozygosity (LOH) analysis:

Polymorphic dinucleotide microsatellite markers, including markers for the *BRCA1* gene (intragenic markers and closely linked markers), for other loci on chromosome 17, for the *BRCA2* gene (closely linked markers), for other loci on chromosome 13, and for the *FHIT* gene on 3p14.2 (intragenic markers), were used to evaluate tissue specimens for evidence of LOH (table 1). These markers were chosen based on their reported high heterozygosity rate [14-16]. Prior to the analysis of the LOH in the tumor and adjacent areas, each microsatellite marker from our panel was first evaluated for informativeness using DNA prepared from the patient's peripheral blood. The tumor tissue of the patients and the surrounding areas to the tumor (normal and benign areas) were then evaluated for LOH at the informative markers. Following microdissection, the cells were immediately incubated in 50μl of digestion buffer (Arcturus) containing proteinase K (1 μg/ml) at 42°C overnight for DNA digestion. After inactivation of proteinase K (95°C for 10 minutes), 1 μl of the digestion material was directly used as a template in a 10 μl PCR reaction. The PCR and cycling conditions

were adapted for each primer set of our microsatellite panel. For each reaction, the genomic DNA obtained from the peripheral blood of the patient was included as normal control for LOH analysis. PCR was performed using a PTC-200 thermo-cycler (MJ Research, Waltham, MA). The primer sets were obtained from Research Genetics Inc. (Huntsville, AL). The forward primers for each set were labeled using one of two fluorescent dyes, HEX or FAM. Allele sizes were determined by electrophoresis of PCR products in 6% denaturing polyacrylamide gels and compared to ROX 500 size standards (Applied Biosystems, Foster City, CA) using an automated sequencer (ABI 377), according to manufacturer's instructions (Applied Biosystems, Foster City, CA). The fluorescent signals from the different size alleles were recorded and analyzed using GENOTYPER version 2.1 and GENESCAN version 3.1 software (Applied Biosystems, Foster City, CA). The presence of LOH was determined by at least two independent observers. For a given informative marker, LOH was defined by a decrease of either peak of at least 50%. The results were read on computer printouts [14]. Each LOH experiment was repeated at least two times, using the same DNA preparations from different PCR reactions, to evaluate the reproducibility of the results.

RESULTS

Using the informative polymorphic dinucleotide microsatellite markers, we evaluated a total of 29 areas showing either normal TDLUs (10 areas) or benign proliferative changes such as sclerosing adenosis (19 areas) from four *BRCA1* and one *BRCA2* positive patients with breast cancer. In addition, tumor tissues were evaluated in each case. Overall, we performed a total of 105 analyses at different loci; of these, LOH was detected in 59 studies (56%). In the normal tissues, 15 of 30 analyses (50%) showed LOH, and in the tissues with proliferative changes, 44 of 75 analyses (59%) showed LOH. Table 2 summarizes the results.

In all 4 cases with a *BRCA1* mutation, LOH at the *BRCA1* gene (marker D17S855) was detected in tumor tissues and in both normal areas and areas with sclerosing adenosis. In case 1 (*BRCA1* +), a sequential loss of the *BRCA1* intragenic marker D17S855 was observed in distant areas, up to 8.7mm from the tumor, as well as in the other three breast quadrants. An interesting finding was the detection of LOH at this marker in the normal contralateral breast, which had been removed prophylactically from this patient (Figure 1 and Table 3). In that same case, LOH at the D17S785 marker on the distal region of 17q was also detected in the tumor and the surrounding normal tissues. In case 2 (*BRCA1* +), not only was LOH at the *BRCA1* marker detected in both the tumor and the surrounding areas with sclerosing adenosis, but also at markers D17S784 and D17S785 located at the distal region of 17q. In case 3 (*BRCA1* +), there was LOH at the D17S855 marker in the benign areas surrounding the invasive ductal carcinoma. This same marker was also lost in normal tissues and tissues with DCIS in the same breast, which was subsequently removed by mastectomy. Markers D17S806 and

D17S579, both flanking the *BRCA1* gene, were also lost, while more distant markers on the p and q arms of chromosome 17 were not lost. In Case 4, in addition to loss of the *BRCA1* gene marker, LOH was also detected at the D17S785 marker located at the distal 17q region in the sclerosing adenosis areas (Figure 2), but not in the normal area surrounding the tumor.

In Case 5 (*BRCA2* +), LOH at the closely linked markers for *BRCA2* was detected in the tumor, in the surrounding areas with normal tissues, and in sclerosing adenosis (Figure 2).

The D3S1300 marker, intragenic to the *FHIT* gene, was lost in most of the cases studied, except in case 1 where no LOH for the *FHIT* gene was detected. In case 3, the invasive tumor and areas surrounding it (both normal and sclerosing adenosis) showed LOH at the *FHIT* locus (Figure 2); no LOH was detected in the DCIS specimen nor in the areas surrounding it.

In all cases where LOH at the *BRCA* loci was detected in the areas showing normal TDLUs or benign proliferative changes, the same allele was the one lost in the tumor, suggesting that the wild type allele was the allele lost by LOH.

DISCUSSION

In familial breast cancers associated with germline mutations in *BRCA1/2* genes, the most common mechanism of inactivation in the tumor is complete loss of the wild-type allele [2-5]. Although this is a highly reproducible observation, the correlation of such an initial LOH event with any specific histopathologic change(s) remains completely unknown. To date, no studies have systematically examined the consequences of inheritance of a mutation in the *BRCA1* or *BRCA2* genes for corresponding, early changes in breast histopathology, nor have studies addressed the correlation of such early abnormalities in the breasts of *BRCA* mutation carriers with either genomic gains, losses, loss of heterozygosity (LOH), or replication error repair instability.

In this study, we have detected LOH at multiple loci, not only in malignant regions in the breasts of *BRCA1* and *BRCA2* mutation carriers with breast cancer, but also in morphologically normal TDLUs and in tissues with benign proliferative changes (e.g. sclerosing adenosis) from these patients. Most of these losses, detected in the normal and benign tissues, were also present in the tumor tissues, suggesting that these non-malignant tissues already harbor significant genetic alterations that may predispose to malignant transformation. Our results support the hypothesis that there is a "field effect" of genomic aberrations, in which some of the genetic changes detected in *BRCA*-associated cancers are also present in the non-malignant areas adjacent to the tumors, as well as in the *BRCA*-positive contralateral prophylactic mastectomy specimens. The concept of "field cancerization" was originally described by Slaughter *et al* in 1953 [17] in their study of oral cancer and was used in reference to the presence of pre-neoplastic

histologic changes at multiple sites. More recent studies have described the molecular changes associated with the multistep development and progression of cancer [18]. In several other studies, morphologically "normal tissues" adjacent to tumors have been shown to harbor molecular changes such as LOH, microsatellite alterations [19,20], chromosomal instability [21], and mutations in the *TP53* gene [22]. Such studies elegantly provide a molecular definition of the old concept of "field cancerization", and may indicate the presence of a field of genetically altered cells which may be at higher risk of cancer transformation.

Additional reports, including our own, support this concept of a "field effect" of genetic alterations in mammary tissue specimens from *BRCA1/2* mutation carriers. Previous studies of both familial and sporadic breast tumors have shown that genetic changes can be detected in morphologically normal appearing breast tissues. LOH has been detected in morphologically normal lobules, adjacent to sporadic breast cancer, suggesting the presence of a "field effect" of preexisting genomic damage in the gland, giving rise to the tumor [19]. LOH was also detected in hyperplasias (usual ductal hyperplasia and atypical ductal hyperplasia) from both cancerous and noncancerous breasts [23-25]. In some cases, the allelic losses observed in these hyperplasias were similar to the ones frequently seen in the breast carcinomas, suggesting a preneoplastic condition and/or a genetically altered precursor cell shared with an adjacent carcinoma. More recently, normal breast tissues adjacent to invasive ductal carcinomas from 12 breast cancer patients (6 monozygotic twin pairs), who were negative for mutations in the *BRCA1/2* genes, were analyzed for LOH on chromosomes 1, 13 and 17. Seventeen LOHs were observed in the normal tissues, 14 of which were present in the

corresponding breast tumor [26]. In addition, two other studies have shown cytogenetic abnormalities in prophylactic mastectomy specimens characterized by hyperplasia, without atypia, from patients with a positive family history of breast cancer (but of unknown *BRCA* status) [27,28].

We have also examined the correlation of *BRCA1* LOH with *FHIT* LOH. Deletions of 3p14 have been observed in benign proliferative breast disease [27,29]; and one report has shown that the *FHIT* gene was homozygously deleted in 2 cases of benign proliferative breast disease associated with 3p14 cytogenetic rearrangements and familial breast cancer [30], suggesting that loss of the *FHIT* gene may be an early event in mammary carcinogenesis. Specifically, we examined whether *FHIT* LOH occurs independent of *BRCA1* LOH, and if so, what are the histopathologic associations with such an observation. Although LOH at *BRCA1* and *FHIT* loci commonly occurred in the same specimens, occasionally we have observed that *BRCA1* LOH occurred in the absence of *FHIT* LOH, but never the reverse. This observation is suggestive of haploid sufficiency of *BRCA1* gene for protection of breast epithelial cells from chromosomal instability. Alternatively, if *FHIT* LOH was common in the absence of *BRCA1* LOH, we might postulate that *FHIT* LOH is an initial or early genetic event in which *BRCA1* carriers are predisposed. In such a scenario, *FHIT* LOH could even predispose the genome to other genetic changes, including *BRCA1* LOH. However, our study does not support this model.

This is the first study to demonstrate genetic changes (LOH) in normal TDLUs and in benign tissues from breast cancer patients that are carriers of mutations in the *BRCA1* and *BRCA2* genes. Such changes may represent the earliest detectable genomic

aberrations that occur during the development and progression of breast cancer in these high-risk patients. We conclude that LOH at the relevant *BRCA* loci is an early event in *BRCA* mutation carriers, and may be detected in non-malignant cells. The significance of this finding needs further evaluation in larger studies, and ultimately may be used as a marker for elevated risk of malignant transformation in these high-risk patients. The identification of such early genetic changes will improve our understanding of the mechanisms of tumorigenesis, and may be useful to develop molecular markers for early detection and diagnosis of hereditary breast cancer in *BRCA1/2* carriers.

Acknowledgements:

REFERENCES

1. Ingvarsson, S. Molecular genetics of breast cancer progression. Semin Cancer Biol., *9*: 277-288, 1999.

2. Cornelis R.S., Neuhausen S.L., Johansson O., Arason A., Kelsell D., Ponder B.A., Tonin P., Hamann U., Lindblom A., Lalle P. High allele loss rates at 17q12-q21 in breast and ovarian tumors from BRCAl-linked families. The Breast Cancer Linkage Consortium. Genes Chrom.Cancer *13*:203-210, 1995.

3. Staff S., Nupponen N.N., Borg A., Isola J.J., and Tanner M.M. Multiple copies of mutant BRCA1 and BRCA2 alleles in breast tumors from germ-line mutation carriers. Genes Chrom.Cancer *28*:432-442, 2000.

4. Nathanson K.N., Wooster R., and Weber B.L. Breast cancer genetics: what we know and what we need. Nat.Med. *7*:552-556, 2001.

5. Osorio A., de la Hoya M., Rodriguez-Lopez R., Martinez-Ramirez A., Cazorla A., Granizo J.J., Esteller M., Rivas C., Caldes T., and Benitez J. Loss of heterozygosity analysis at the BRCA loci in tumor samples from patients with familial breast cancer. Int.J.Cancer *99*:305-309, 2002.

6. Xu X., Wagner K.U., Larson D., Weaver Z., Li C., Ried T., Hennighausen L., Wynshaw-Boris A., and Deng C.X. Conditional mutation of *BRCA1* in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat.Genet. *22*:37-43, 1999.

7. Negrini M., Monaco C., Vorechovsky I., Ohta M., Druck T., Baffa R., Huebner K., and Croce C.M. The *FHIT* gene at 3p14.2 is abnormal in breast carcinomas. Cancer Res. *56*:3173-3179, 1996.

8. Campiglio M., Pekarsky Y., Menard S., Tagliabue E., Pilotti S., and Croce C.M. FHIT loss of function in human primary breast cancer correlates with advanced stage of the disease. Cancer Res. *59*:3866-3869, 1999.

9. Gatalica Z., Lele S.M., Rampy B.A., and Norris B.A. The expression of Fhit protein is related inversely to disease progression in patients with breast carcinoma. Cancer *88*:1378-1383, 2000.

10. Bergthorsson J.T., Eiriksdottir G., Barkardottir R.B., Egilsson V., Arason A., and Ingvarsson S. Linkage analysis and allelic imbalance in human breast cancer kindreds using microsatellite markers from the short arm of chromosome 3. Hum. Genet. *96*:437-443, 1995.

11. Ingvarsson S., Geirsdottir E.K., Johannesdottir G., Sigbjornsdottir B.I., Eiriksdottir G., Ragnarsson G., Agnarsson B.A., Gudmundsson J., Jonasson J.G., Sigurdsson A., Egilsson V., and Barkardottir R.B. High incidence of loss of heterozygosity in breast tumors from carriers of the BRCA2 999del5 mutation. Cancer Res. *58*:4421-4425, 1998.

12. Ingvarsson S., Agnarsson B.A., Sigbjornsdottir B.I., Kononen J., Kallioniemi O.P., Barkardottir R.B., Kovatich A.J., Schwarting R., Hauck W.W., Huebner K., and McCue P.A. Reduced Fhit expression in sporadic and BRCA2-linked breast carcinomas. Cancer Res. *59*:2682-2689, 1999.

13. Emmert-Buck M.R., Bonner R.F., Smith P.D., Chuaqui R.F., Zhuang Z., Goldstein S.R., Weiss R.A., and Liotta L.A. Laser capture microdissection. Science *274*:998-1001, 1996.

14. Kerangueven F., Noguchi T., Coulier F., Allione F., Wargniez V., Simony-Lafontaine J., Longy M., Jacquemier J., Sobol H., Eisinger F., and Birnbaum D. Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. Cancer Res. *57*:5469-5474, 1997.

15. Phelan C.M., Borg A., Cuny M., Crichton D.N., Baldersson T., Andersen T.I., Caligo M.A., Lidereau R., Lindblom A., Seitz S., Kelsell D., Hamann U., Rio P., Thorlacius S., Papp J., Olah E., Ponder B., Bignon Y.J., Scherneck S., Barkardottir R., Borresen-

Dale A.L., Eyfjord J. Theillet C., Thompson A.M., Devilee P., and Larsson C. Consortium study on 1280 breast carcinomas: allelic loss on chromosome 17 targets subregions associated with family history and clinical parameters. Cancer Res.*58*:1004-1012, 1998.

16. Euhus D.M., Maitra A., Wistuba II., Alberts A., Albores-Saavedra J., and Gazdar A.F. Loss of heterozygosity at 3p in benign lesions preceding invasive breast cancer. J.Surg.Res.*83*:13-18, 1999.

17. Slaughter D.P., Southwick, H.W., and Smejkal, W. "Field cancerization" in oral stratified squamous epithelium. Cancer (Phila.), *6*:693-968, 1953.

18. Fearon E.R. and Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. *61*:759-767, 1990

19. Deng G., Lu Y., Zlotnikov G., Thor A.D., and Smith H.S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science *274*:2057-2059, 1996.

20. Tabor M.P., Brakenhoff R.H., van Houten V.M., Kummer J.A., Snel M.H., Snijders P.J., Snow G.B., Leemans C.R., and Braakhuis B.J. Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. Clin Cancer Res. *7*:1523-1532, 2001.

21. Hittelman W.N. Genetic instability in epithelial tissues at risk for cancer. Ann N Y Acad Sci. *952*:1-12, 2001.

22. Brennan J.A., Mao L., Hruban R.H., Boyle J.O., Eby Y.J., Koch W.M., Goodman S.N., and Sidransky D. Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. N Engl J Med. *16*;332(7):429-435, 1995.

23. O'Connell P., Pekkel V., Fuqua S., Osborne C.K., and Allred D.C. Molecular genetic studies of early breast cancer evolution. Breast Cancer Res.Treat. *32*:5-12, 1994.

24. O'Connell P., Pekkel V., Fuqua S.A., Osborne C.K., Clark G.M., and Allred D.C. Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. J. Natl.Cancer Inst. *90*:697-703, 1998.

25. Washington C., Dalbegue F., Abreo F., Taubenberger J.K., and Lichy J.H. Loss of heterozygosity in fibrocystic change of the breast: genetic relationship between benign proliferative lesions and associated carcinomas. Am. J. Pathol. *157*:323-329, 2000.

26. Forsti A., Louhelainen J., Soderberg M., Wijkstrom H., and Hemminki K. Loss of heterozygosity in tumour-adjacent normal tissue of breast and bladder cancer. Eur. J.Cancer *37*:1372-1380, 2001.

27. Petersson C., Pandis N., Mertens F., Adeyinka A., Ingvar C., Ringberg A., Idvall I., Bondeson L., Borg A., Olsson H., Kristoffersson U., and Mitelman F. Chromosome aberrations in prophylactic mastectomies from women belonging to breast cancer families. Genes Chrom.Cancer *16*:185-188, 1996.

28. Teixeira M.R., Pandis N., Gerdes A.M., Dietrich C.U., Bardi G., Andersen J.A., Graversen H.P., Mitelman F., and Heim S. Cytogenetic abnormalities in an in situ ductal carcinoma and five prophylactically removed breasts from members of a family with hereditary breast cancer. Breast Cancer Res. Treat. *38*:177-182, 1996.

29. Dietrich C.U., Pandis N., Teixeira M.R., Bardi G., Gerdes A.M., Andersen J.A., and Heim S. Chromosome abnormalities in benign hyperproliferative disorders of epithelial and stromal breast tissue. Int.J.Cancer *60*:49-53, 1995.

30. Panagopoulos I., Pandis N., Thelin S., Petersson C., Mertens F., Borg A., Kristoffersson U., Mitelman F., and Aman P. The FHIT and PTPRG genes are deleted in benign proliferative breast disease associated with familial breast cancer and cytogenetic rearrangements of chromosome band 3p14. Cancer Res. *56*:4871-4875, 1996.

Table 1. The panel of microsatellite markers used. The chromosomal location, the expected size range of the amplified PCR product and the percent heterozygozyty for ecach marker are indicated.

	Site	Size (bp)	Heterozygozity (%)
D17S786	17p12	135-157	77
TP 53	17p13	230	90
D17S849	17p13	215-253	67
D17S250	17q11.2-q12	151-169	91
D17S806	17q21	153-185	91
D17S855	17q21.2 (*BRCA1*)	145	82
D17S579	17q21.3	111-133	87
D17S785	17q24	181-207	84
D17S784	17q25	226-238	79
D13S289	13q12.1	260-276	74
D13S153	13q14.1-q14.3	212-236	82
D13S137	13q14.3	113-135	84
D13S173	13q32-q34	166-178	84
D3S1300	3p21.1-14.2 (*FHIT*)	217-241	83
D3S1481	3p14.2 (*FHIT*)	104	83

Table 2. Summary of the results showing the number of analyses performed and the percent of LOH detected.

	Total # of analyses	LOH found
Total	105	59 (56%)
Normal TDLU	30	15 (50%)
Proliferative Changes	75	44 (59%)

Table 3. Summary of LOH analyses at the *BRCA1* marker (D17S855) in tissues isolated from case 1 from different areas of the breast with tumor and of the contralateral breast removed prophylacticaly.

	Quadrant	Tissue	Distance from Tumor	LOH at D17S855
Breast with tumor	Upper Outer Quadrant	Tumor		LOH
		Sclerosing Adenosis	0.1 mm	LOH
		Sclerosing Adenosis	0.3 mm	LOH
		Normal	0.8 mm	LOH
		Normal	3.5 mm	No LOH
		Sclerosing Adenosis	6 mm	LOH
		Normal	8.7 mm	LOH
	Upper Inner Quadrant	Sclerosing Adenosis		LOH
	Lower Outer Quadrant	Sclerosing Adenosis		LOH
	Lower Inner Quadrant	Sclerosing Adenosis		LOH
Contralateral breast- no tumor		Normal		No LOH
		Sclerosing Adenosis		LOH

Legends for illustrations

Figure 1: The left top image shows a histologically normal terminal duct lobular unit (H&E, 20X) adjacent to the tumor, from a *BRCA1* positive patient with cancer. The left middle image shows the lobule after laser capture microdissection (LCM). A majority of the epithelial tissue has been microdissected. The left bottom image shows an area with sclerosing adenosis (arrow head) (H&E, 20X), adjacent to the tumor from the same patient. The tracings on the right show the LOH analysis using the D17S855 *BRCA1* intragenic marker, performed on tissues microdissected from the same patient's specimens. Tissues studied were isolated using LCM. From top to bottom, studies in blood (Bl) show the marker to be heterozygote. Loss of heterozygosity is detected in the tumor (T). The same allele is also lost in normal lobular tissue adjacent to the tumor (N1) and in an area with sclerosing adenosis adjacent (SA1) to the tumor. In the contralateral breast, normal tissues (N2) do not show LOH, while an area with sclerosing adenosis (SA2) shows LOH for the *BRCA1* marker.

Figure 2: Figure 2 shows other examples of LOH at the following loci (from left to right): D13S153, D17S785, and D3S1300 in cases 5, 4, and 3, respectively. In each panel, the top tracing shows the analysis in the blood (Bl), the middle tracing shows the analysis in the tumor (T) and in the bottom tracing the analysis in an area with sclerosing adenosis.

Bl

144.57 152.85

T

143.64

N1

144.44
152.76

SA1

143.64

N2

143.64 152.15

SA2

144.73
152.94